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ORIGINAL PAPER

Highly efficient analysis of underivatized carbohydrates using monolithic-silica-based capillary hydrophilic interaction (HILIC) HPLC

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Abstract A polyacrylamide (PAAm)-modified monolithic silica capillary column of increased phase ratio, 200T-PAAm, for hydrophilic interaction liquid chromatography (HILIC) was prepared. The column showed high separation efficiency, with a theoretical plate height H=7–20 μm at a linear velocity, u=1–7 mm/s. From a kinetic plot analysis, it was expected that the monolithic column could provide three times faster separation than particle-packed HILIC columns under a pressure limit at 20 MPa. HILIC coupled with electrospray ionization (ESI)–mass spectrometry (HILIC-ESI-MS) using the 200T-PAAm column was employed for the analysis of underivatized carbohydrates to achieve fast and efficient separations of mixtures containing mono-, di-, and trisaccharides within 5 min. Under single MS full scan mode, 200 pg of oligosaccharides was detected by the system. The limit of detection (LOD) of the LC-ESI-MS/MS system was determined using selected reaction monitoring (SRM) to be as low as 3.2 ng/mL (attomol level) for nonreducing saccharides. The system was successfully applied to the detection of disaccharides in extracts of plant, such as corn, soybean, and *Arabidopsis thaliana*.

Keywords Monolithic silica · HILIC · Polyacrylamide · Underivatized carbohydrates · HILIC-ESI-MS

Introduction

Carbohydrates have been challenging targets for analytical chemists because of difficulties in both their separation and detection. Due to the chirality at many carbon atoms, the possibility of anomers, the existence of structures containing branching, crosslinking, and several differences in three-dimensional structures, the number of isomers of carbohydrates is huge, and their separation is difficult. However, two- and three-dimensional carbohydrate mapping in detail is already available [1]. The hydroxyl groups of carbohydrates are very weak acids, and can be ionized under strongly alkaline conditions (pH>12). They are separated by anion-exchange chromatography under basic conditions [2]. Aminopropyl-modified silica columns have been used for the separation of carbohydrates [3–5] using hydrophilic interaction chromatography (HILIC) mode [6, 7] although

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this type of column sometimes has the problem of higher background intensity due to the limited stability of the phase in aqueous mobile phase [8], and thus it has low sensitivity for the detection of carbohydrates when they are applied to liquid chromatography–mass spectrometry (LC-MS) systems. Recently, the use of other HILIC methods for the separation of carbohydrates has been studied owing to the development of columns for these separation modes [9–11]. For this separation mode, silica particles are modified with aminopropyl groups as above, amide groups from polysuccinimide [9], zwitterionic [12], and amide groups [13–15]. A capillary column packed with amide silica was used in a liquid chromatography coupled with electrospray ionization (ESI)–mass spectrometry (LC-ESI-MS) system to detect native oligosaccharides at low femtomolar sensitivity [16]. A graphitized carbon column has also been widely used for the separation of underivatized carbohydrates [17–20], and in some cases, low femtomolar limits of detection were achieved [20]. Recently, aqueous normal-phase (ANP) mode HPLC was invented using a silica-based column, in which the alkyl-group-based stationary phases were attached to hydride-type silica [21, 22]. In this separation mode, both nonpolar and polar compounds were retained in the column, and it will be a unique separation tool for metabolomics. Since they have no significant UV–Vis absorption due to the lack of chromophores in their native forms, the detection of carbohydrates has limited options [23].

It is desirable to avoid the derivatization of carbohydrates for quantitative measurements, because highly efficient and selective derivatization methods are limited. In HPLC systems, the underivatized carbohydrates can be detected by indirect UV detection [24–27], electrochemical detection [28, 29], and refractive index (RI) detection [30]. Until the late 1990s, the use of ESI-MS systems for the detection of underivatized carbohydrates was limited when compared to matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-MS) systems. The main problem of the ESI-MS system is its sensitivity; generally, peptides can be detected in the low femtomolar range, whereas carbohydrates need a picomolar sample [31]. The invention of nano-ESI-MS improved the detection limits of underivatized oligosaccharides to 100 attomol of sample using ion-trap mass spectrometry (IT-MS) with the direct infusion of a sample solution [32].

Capillary electrophoresis (CE) has also been studied widely for the separation of carbohydrates [33]. Underivatized and derivatized carbohydrates were detected by direct absorption at 195 nm [34], pulsed amperometric detection [35, 36], electrochemical detection [37, 38], and indirect absorption [27, 39]. The use of an alkaline borate buffer provides 2–50 times more sensitive detection at 195 nm [34]. In general, the use of this method for the analysis of underivatized carbohydrates is still limited; however, there is an example of very high separation efficiency of *N*-acetylneuramic acid polymers and hyaluronic acid [40]. A polymer-based monolithic column containing cyano functionality generated high efficiency, 200,000 plates/m, for the separation of native mono- and oligosaccharides by a capillary electrochromatography (CEC), combined with an ESI-MS system [41]. CE-MS has been reported to be a very powerful tool to analyze metabolomes, including neutral and phosphorylated carbohydrates quantitatively up to the low attomol range [42]. In CE separation, the separation efficiency for underivatized and uncharged saccharides seems to be low when compared with properly derivatized saccharides.We describe here the high performance of a monolithic silica capillary column for HILIC even in an analytical system possessing a large extra-column effect, such as LC-ESI-MS. Starting from a monolithic silica support with a higher phase ratio, which was shown to afford higher column efficiency down to H= 4–5 μm [43], a polyacrylamide-coated column was prepared by an on-column polymerization. The use of the monolithic silica support resulted in higher separation efficiency than a previously reported polyacrylamide-coated monolithic silica column [44]. The separation and detection of underivatized carbohydrates using the high-separation-efficiency column was performed by LC-ESI-MS and LC-ESI-MS/MS systems to provide subpicomol-level detection for the former system, and sub-femtomol-level detection for the latter.

Experimental

Chemicals

All carbohydrate samples were purchased from Sigma (St. Louis, MO, USA). LC-MS grade ammonium acetate was obtained from Fluka (Ronkonkoma, NY, USA), and dissolved in water to a concentration of 13 mM (controlled at pH=5.5 with acetic acid) for a buffer solution throughout the experiment. Guanosine was obtained from Wako (Osaka, Japan). LC-MS grade acetonitrile (CH_3CN) was purchased from J. T. Baker (Phillipsburg, NJ, USA), and used without further purification. Water was purified by a Milli-Q Gradient system (Millipore, Billerica, MA, USA) after passing through cation-exchange resin.

Column preparation

A monolithic silica capillary column (267 mm×200 μm ID) with a higher phase ratio was prepared in-house by a previously reported method [43]. The monolithic silica column was modified with (3-methacrylamidopropyl)triethoxysilane, followed by a polymer-coating using acrylamide by a previously reported procedure with a minor modification [44]. The column is denoted by an abbreviation, 200T-PAAm, in which 200, T, and PAAm stand for the inner diameter of the column (200 μm), type of silica (prepared from tetramethoxysilane), and the immobilized stationary phase (polyacrylamide), respectively.

HPLC characterization of the 200T-PAAm column

Chromatographic characterization was carried out by an LC-10AD VP pump (Shimadzu, Kyoto, Japan). The mobile phase was CH_3CN/13 mM ammonium acetate (NH_4OCOCH_3) (pH=4.7) (80:20, v/v). Guanosine dissolved in the mobile phase (1 mg/mL) was employed as a sample. UV detection was performed using a UV–Vis detector (CE1575, Jasco, Hachioji, Japan) at a wavelength λ=254 nm with on-column detection. Column dead time, t_0 was estimated by a peak due to a solvent.

HPLC-ESI-MS apparatuses

Chromatographic separation was carried out by an Agilent 1100 (Agilent Technologies, Santa Clara, CA, USA) with a split flow. Mobile phase A was CH_3CN and B was an aqueous buffer solution of 13 mM NH_4OCOCH_3 (pH=5.5). The mobile phase was introduced to the injector after the split. The samples were injected using a sample injector with a 50 nL internal loop (VICI, C4-0004-.05, Schenkon, Switzerland). A linear ion-trap mass spectrometer, Finnigan LTQ (Thermo Fisher Scientific, Waltham, MA, USA) with an electrospray ionization unit for the nanosource with a PicoTip™ EMITTER SilicaTip™, FS360-50-30-D (New Objectives, Woburn, MA, USA) was connected to an empty capillary (200 mm×50-μm ID) following the monolithic column, using a metal union. This was employed in negative mode to detect the underivatized carbohydrates. t_0 was estimated by a peak around 1 min due to an unretained compound. In the calculation of split ratio, the porosity of the column was estimated as 0.8. The ionization voltage was 2 kV. The MS conditions were as follows: capillary voltage 3.2 kV, cone voltage 30 V, extractor 1 V, and rf lens 0.3 V. The source temperature was set to 100 °C, and desolvation to 350 °C. The desolvation gas flow was set to 350 L/h, and cone gas to 50 L/h. The MS scan span was 160–800 m/z, and the scan time 2.2 s with a 0.1 s interscan time.

An experiment for the relationship between ln$k$ values of oligosaccharides and the CH_3CN concentrations was performed by an LC-quadrupole-time-of-flight (Q-TOF) system consisting of an LC-20AD pump (Shimadzu) with the injector with a 50 nL internal loop and a micro mass spectrometer (Micromass, Beverly, MA, USA) equipped with a nanoelectrospray probe. The abovementioned ESI chip was connected to the monolithic column using a metal union. The typical operating conditions for the Q-TOF micro mass spectrometer were as follows: spray voltage 2.8 kV (negative mode), sample cone voltage 40 V, and source temperature 20 °C. The collision gas pressure was activated at 10 V. Each spectrum was integrated with a scan duration of 0.52 s and interscan delay of 0.10 s.

Sample preparation

All commercially available carbohydrates were dissolved in water to a concentration of 1.0 mg/mL as the stock solutions. For the isocratic HPLC separation, 200 μL each from the stock solutions of sedoheptulose, sucrose, trehalose, and raffinose were mixed together to prepare a standard sample. In the gradient mode separation, 200 μL each from the stock solutions of glucose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were mixed together to prepare a standard sample and then the mixture was diluted with water. This mixture was again diluted with CH_3CN to prepare 20 μg/mL and 4 μg/mL sample solutions (CH_3CN/water = 90:10), respectively. Plant extracts from dried mature corn kernel, fresh frozen young soybean seeds, and fresh frozen rosette leaves of *Arabidopsis thaliana* were prepared by the following standard operating procedure.

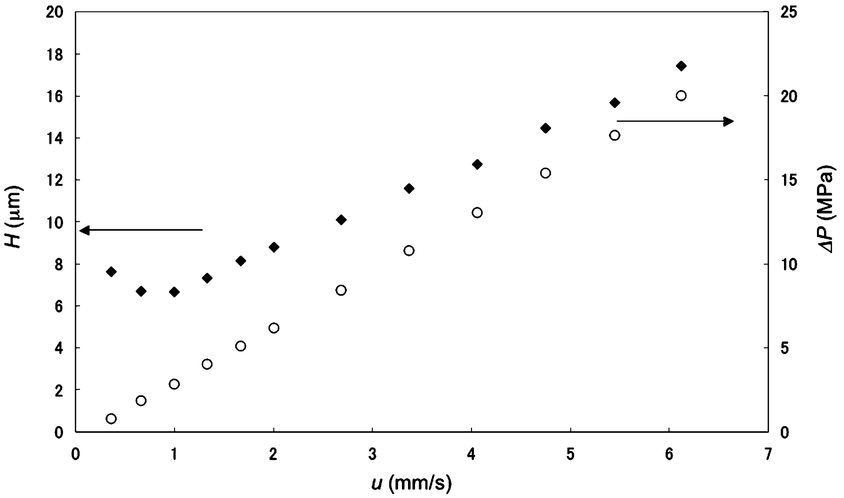
Tissue processing Fresh frozen tissues or dry lyophilized tissues were kept in 2 mL round-bottomed Eppendorf tubes, equipped with one 3-mm-ID steel ball, and were homogenized with a Retsch ball mill for 30 s at 25 s^{-1}.

Extraction of corn and soybean Ground tissue powder was weighed into aliquots of 50 mg fresh weight (5 mg dry weight) and kept in liquid nitrogen between homogenization and extraction. The extraction solvent was prepared by mixing isopropanol/acetonitrile/water at a ratio of 3:3:2 (v/v/v) and degassing this mixture by directing a gentle stream of nitrogen through the solvent for 5 min. The solvent was cooled to −20 °C prior to extraction. A 1 mL aliquot of cold solvent was added, vortexed for 10 s, and shaken at 4 °C for 5 min to extract the metabolites and simultaneously precipitate the proteins. After centrifugation at 12,800×g for 2 min, 90% of the supernatant was removed, taking care not to remove any residues from the pellet.

Extract concentration for corn and soybean The supernatant was separated into two equal aliquots and concentrated to dryness in a vacuum concentrator at room temperature for 4 h.

Extract storage The dried extracts can be kept under nitrogen at −80 °C for up to 4 weeks. In the study

Fig. 1 The van Deemter plot with a column backpressure plot for the monolithic silica column 200T-PAAm. Column 200T-PAAm, 285 mm×200-μm ID; mobile phase 80% CH_3CN (13 mM NH_4OCOCH_3, pH=4.7); temperature 30 °C; detection UV–Vis, λ=254 nm; solute guanosine (k=3.8); sample volume 1.0-μL injection (split). Plate height (♦), backpressure (○)



presented here, the extracts were immediately resuspended in 50 mL of 50% aqueous acetonitrile for injection into the HILIC–ion-trap mass spectrometer.

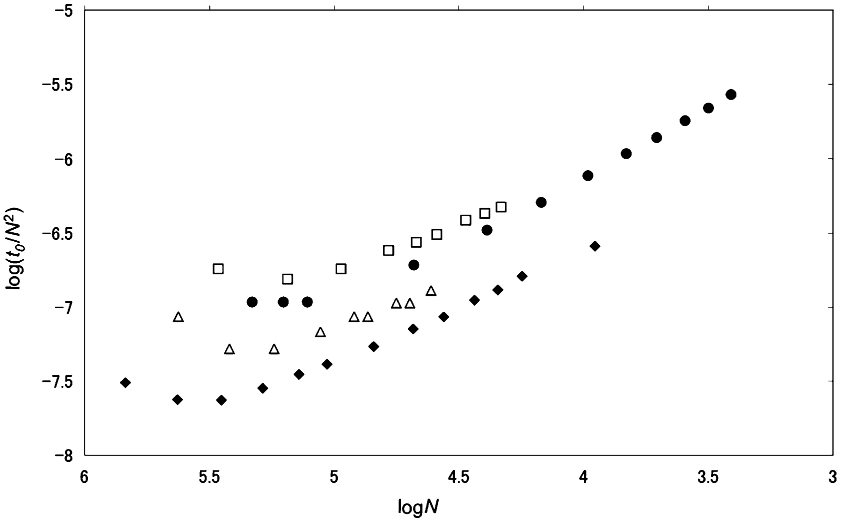
Sample preparation including extraction of rosette leaves of Arabidopsis thaliana A total of 10 mg of ground tissue powder was weighed into the Eppendorf tubes, 1 mL of pure water was added, and shaken at 4 °C for 30 min to extract the metabolites and simultaneously precipitate the proteins. After centrifugation at 12,800×g for 2 min, 90% of the supernatant was removed, and diluted tenfold with water. The solution was then diluted with CH_3CN in the ratio of 20:80 (80% CH_3CN solution).

Data analysis The MS data were processed with MassLynx software (Waters). The retention times of the carbohydrates were recorded manually according to their mass (extracted single ion recording (SIR)-MS chromatogram), and entered into MS Excel.

Results and discussion

A monolithic silica capillary column for the HILIC, 200T-PAAm, was prepared by the on-column polymerization reaction of acrylamide in a capillary column containing monolithic silica of a higher phase ratio [43] as compared to previous materials [45, 46]. The chromatographic efficiency of the 200T-PAAm column was examined at several different flow rates using guanosine (retention factor, k=3.8) as a test sample, taking into account the extra-column band-broadening effect [47]. The column efficiency of 200T-PAAm was found to be much higher than the PAAm-coated monolithic silica column previously

Fig. 2 Kinetic plot of $\log(t_0/N^2)$ versus $\log N$ with the assumed maximum pressure of 20 MPa for the (♦) MS-200T-PAAm, (●) the ZIC-HILIC, (□) the TSK-GEL Amide-80, 5 μm, and (△) the TSK-GEL Amide-80, 3 μm columns. Chromatographic conditions for TSK-GEL Amide-80 columns are as follows: column TSK-GEL Amide-80, 250 mm× 4.6 mm ID, 5 μm and 150 mm× 4.6 mm ID, 3 μm; mobile phase 75% CH_3CN/H_2O; temperature 40 °C; detection RI; solute mannitol (k=2.5); sample volume 10 μL injection. For other chromatographic conditions, see Fig. 1



reported, which provided H=16 μm at linear velocity, u, 1.0 mm/s flow rate [44]. Figure 1 shows the van Deemter plot along with the column backpressure at each flow rate. The optimum linear velocity was shown to be around u=1.0 mm/s with a theoretical plate height H=7 μm. This result was slightly worse than in the reversed-phase (RP) HPLC system using monolithic silica capillary columns with a similar phase ratio domain size [43]. At a flow rate of 6 mm/s, the present column backpressure reached 20 MPa, and H increased to 18 μm. These results were compared with those of a particle-packed column, ZIC-HILIC (5 μm, 150 mm×4.6 m ID), available from their product report [48]. The optimal flow rate of the column is 0.2–0.5 mL/min, i.e., u=0.31–0.77 mm/s with H=12 μm, and the H–u curve slopes increase steeply with the flow rate. At higher linear velocity u=7.7 mm/s, the column backpressure reached 12.5 MPa, and the H value increased drastically up to 60 μm. This result was obtained using cytocine (k=1.3) as a sample, and the efficiency of the HILIC system should be more advantageous at such a small retention region versus an analyte with a larger retention, such as the guanosine (k=3.8) chosen in our experiment.

In order to compare the kinetic performance of three particle-packed columns including the ZIC-HILIC column and two TSK-GEL Amide-80 columns (250 mm×4.6 mm ID, 5 μm) and (150 mm×4.6 mm ID, 3 μm), against the monolithic silica column 200T-PAAm, the log(t_0/N^2) value was plotted against log$N$ as shown in Fig. 2 [49], with a maximum pressure of 20 MPa, which is the upper limit in most HPLC applications. The plot indicates the largest theoretical plate number N attainable under a certain backpressure drop, and the separation time, in terms of column dead time, t_0. Thus, it is easy to find the limits of the separation efficiency considering the separation time under a constant backpressure. At all regions of log$N$, the monolithic silica column can generate the same separation efficiency as particle-packed columns, and is roughly three times faster than the ZIC-HILIC column. For example, the 200T-PAAm column can provide N=9,000 with t_0=20 s, and the limit of efficiency is estimated at N=280,000 with t_0=1,900 s, whereas the particle-packed columns generate N=3,000 with t_0=20 s, and N=9,600 with t_0=70 s for the ZIC-HILIC column, and in the maximum efficiency is estimated at N=210,000 although t_0 reaches 4,900 s.

Recently, an amide-modified silica column packed with 3-μm particles has been released, and it generates H=7 μm at u=3 cm/min, i.e., 0.5 mm/s at 40 °C. At a higher linear velocity, u=1.0 and 2.0 mm/s, H=7.5 and 11 μm were generated for mannitol at the same temperature. At 25 °C, N=17,000 and H=8.8 μm were obtained for mannitol (k=2.5) at a 1.0 mL/min flow rate [50]. The present monolithic silica PAAm column showed slightly higher column efficiency with a much higher permeability similar to the columns packed with 5-μm particles. By the comparison of the kinetic plots in Fig. 2, the TSK-GEL Amide-80 packed with 3 μm particles possessed better kinetic performance than the columns packed with 5 μm particles, but the 200T PAAm monolithic column can generate the same separation efficiency 1.7 times faster than the columns Amide-80 packed with 3 μm particles.

In RP mode, the monolithic column was estimated to generate N=100,000 with t_0=100 s at 40 MPa, whereas t_0=3,160 s, 250 s, and 320 s are required to provide N=100,000 under the same conditions using columns packed with 2-, 3-,

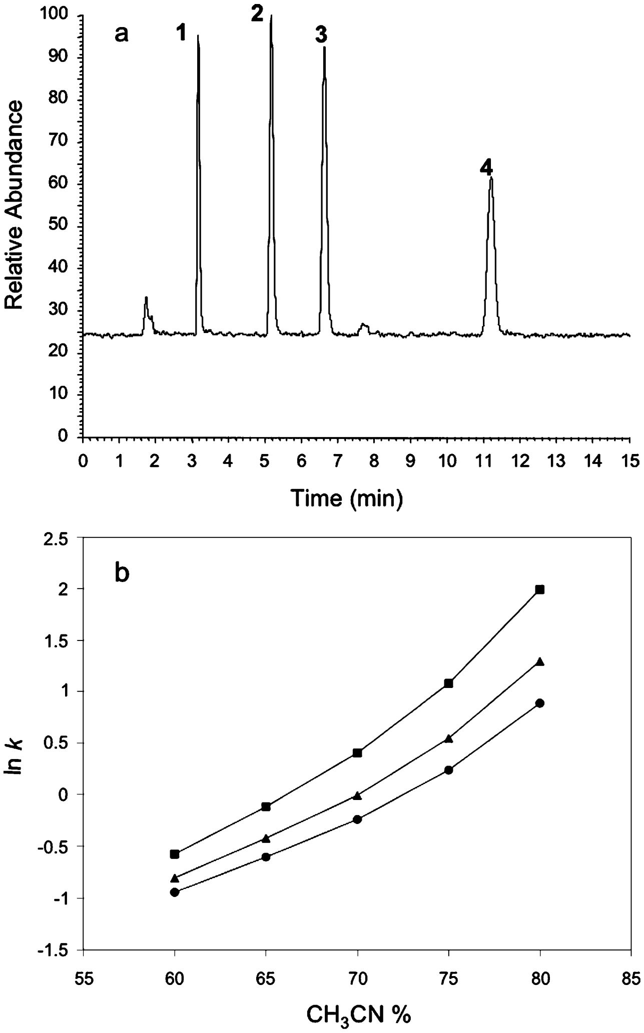


Fig. 3 **a** LC-ESI-MS total ion chromatogram of a mixture of mono-, di-, and trisaccharides obtained in 80% CH_3CN (13 mM NH_4OCOCH_3). Column 200T-PAAm, 267 mm×200 μm ID; flow rate 0.2 mL/min; linear velocity 2.7 mm/s (split ratio 48:1); detection 2.0 kV negative ESI–ion-trap MS; solutes *1* sedoheptulose, *2* sucrose, *3* trehalose, *4* raffinose. **b** Plots of the ln$k$ values of oligosaccharides in various CH_3CN/water mixtures on a 200T-PAAm column. Column 195 mm×200 μm ID; temperature 30 °C, detection 2.8 kV negative ESI-TOF-MS; solutes sucrose (●), trehalose (▲), raffinose (■) (each 100 μg/mL); injection volume 50 nL

and 5 μm particles, respectively [45]. Thus, the N^2/t_0 value of the monolith column was three times greater than that of columns packed with 5 μm particles. A PAAm-coated monolithic silica capillary column of the older type was reported to separate carbohydrates derivatized with 2-aminopyridine (PA) efficiently [44], but there was no example of its application to an HPLC-MS system. To confirm that a monolithic silica capillary column modified with polyacrylamide is suitable for the separation and detection of underivatized carbohydrates by an HPLC-ESI-MS system, a mixture of sedoheptulose, sucrose, trehalose, and raffinose was employed as a test sample. As expected, these carbohydrates were eluted in order of molecular size from small to large, as shown in Fig. 3a. The relationship between the chromatographic retention factor k and the CH_3CN concentration in the mobile phase was examined over the range from 60 to 80% CH_3CN/13 mM NH_4OCOCH_3 buffer to confirm that the system also shows typical HILIC retentivity, as exhibited in Fig. 3b. For this experiment, a time-of-flight (TOF) MS was used as the detector. The chromatographic retention of these carbohydrates on the column were k=1–6, but decreased drastically to k=1 in 70% CH_3CN, and finally reached k=0.5 in 60% CH_3CN. In the case of the former monolithic silica coated with PAAm, the retention factor of guanosine was k=0.5 in 80% CH_3CN, and it was apparently smaller than that of the present 200T-PAAm column due to the difference in the phase ratio of these two columns. The column separation efficiency of the 200T-PAAm was estimated at 12,000–14,000 theoretical plates, and the theoretical plate height could be calculated as H=19–22 μm. The H values are significantly larger than H=7 μm from the fundamental column characterization using a UV detector as shown in Fig. 1, presumably due to the large extra-column effect at the ESI interface between the LC and MS. The previous HILIC monolithic silica capillary column provided H=18 μm for solutes of small retention (k<1) under the optimal flow rate [44].

The application of capillary HILIC to separate underivatized monosaccharides is still limited. In an example of the separation, L-fucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, N-acetylneuraminic acid, and D-glucuronic acid were separated using a column modified with an amide-type silica, TSK-GEL Amide-80 [15]. Higher separation efficiency was observed at 60 °C than at 40 or 50 °C, and the plate numbers for L-fucose, N-acetylneuraminic acid, and D-glucuronic acid were N=1,700–3,000 even at 60 °C with k values of 0.9–18. The H values were estimated as 80–150 μm, although the detection method, evaporative light scattering detection (ELSD), might have a significantly large extra-column effect to yield larger theoretical plate heights.

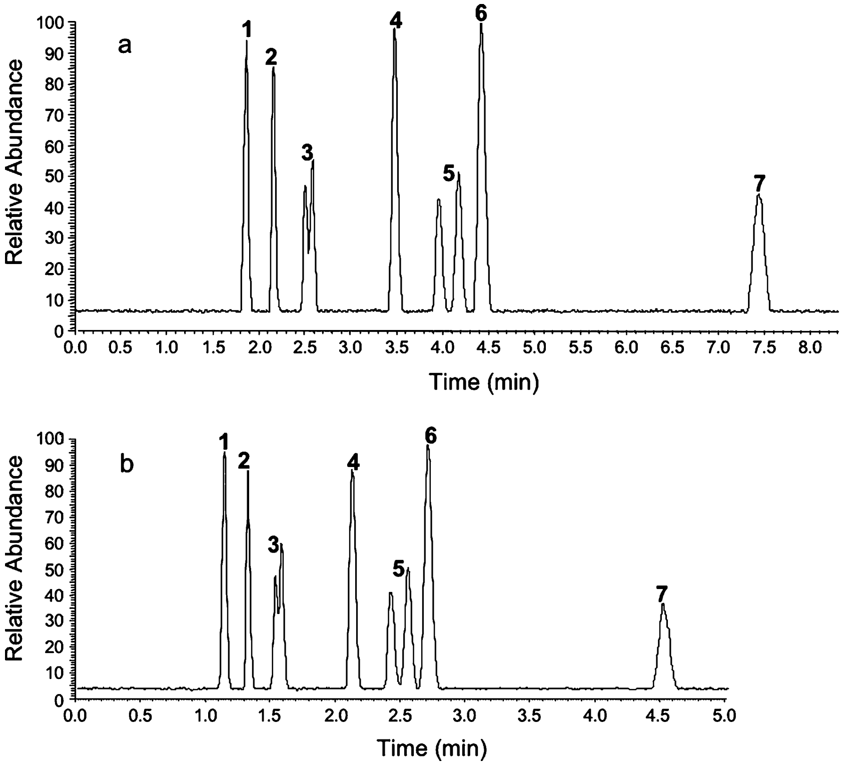


Fig. 4 LC-ESI-MS base peak chromatograms of a mixture of mono-, di-, and trisaccharides obtained in 80% CH_3CN (13 mM NH_4OCOCH_3), flow rate 0.2 mL/min; linear velocities **a** 3.9 mm/s (split ratio 34:1), and **b** 7.6 mm/s (split ratio 17:1); column 200T-PAAm, 267 mm×200 μm ID; detection 2.2 kV negative ESI–ion-trap MS; solutes *1* ribose, *2* sedoheptulose, *3* glucose, *4* sucrose, *5* maltose, *6* trehalose, *7* raffinose

Detection methods applicable to underivatized saccharides such as ELSD, RI, electrochemical detection, and ESI-MS tend to cause considerable band broadening due to the large extra-column effect, and thus it is difficult to obtain high separation efficiency, though a CEC-ESI-MS system produced a high separation efficiency around N=60,000 for the analysis of underivatized oligosaccharides [41]. This circumstance is also found in the classical HPLC system using an amino-silica column.

Figure 4 shows the results of the high-speed separation of ribose, sedoheptulose, glucose, sucrose, maltose, trehalose, and raffinose at linear velocities of u=3.9 mm/s (Fig. 4a) and u=7.6 mm/s (Fig. 4b) in 80% CH_3CN/13 mM NH_4OCOCH_3. In the case of the latter separation, the last peak raffinose was eluted at 4.5 min and the disaccharides were eluted at around 2 min. At a higher linear velocity, decreased column efficiency at u=3.9 mm/s was observed with plate numbers of 15,000–19,000, and 12,000–14,000 at u=7.6 mm/s, except for the peak of ribose which showed worse efficiency. The extra-column effect and anomerization could be reasons for the band broadening of the less strongly retained solutes such as ribose. One of the significant advantages of LC using monolithic silica columns over electrophoresis and related separation technologies is the potential of the fast separation of carbohydrates. In the case of glucose and maltose, peak splitting due to anomer separation was observed. The other samples are nonreducing carbohydrates and did not show any peak splitting. The peak splitting due to anomer separation of the carbohydrates often causes peak broadening and tailing which leads to low separation efficiencies [51]. In the present chromatogram, the peak shape of each anomer was symmetrical, and peak tailing was not significant.

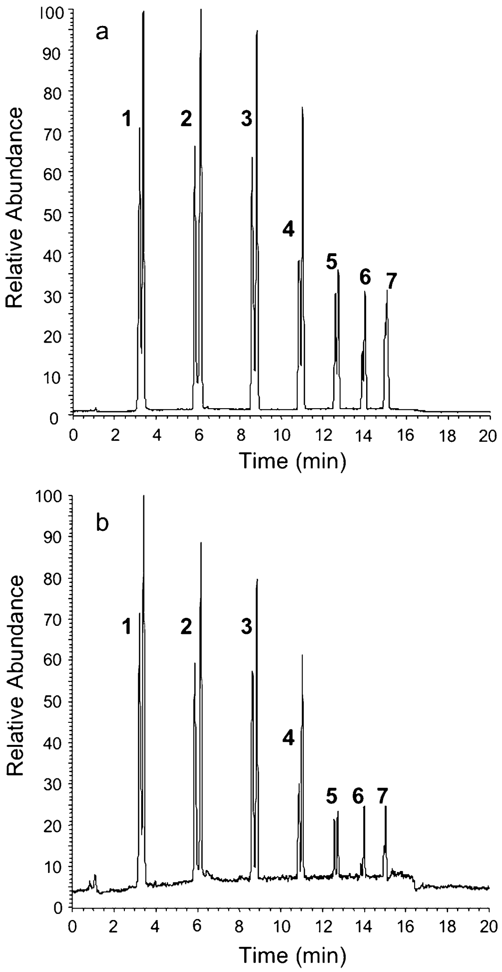


Fig. 5 LC-ESI-MS base peak chromatogram of a mixture of maltooligosaccharides: mobile phase 90–60% CH_3CN (13 mM NH_4OCOCH_3) in a 15 min linear gradient; flow rate 0.2 mL/min; column (**a** and **b**) 200T-PAAm, 267 mm×200 µm ID; linear velocity 4.2 mm/s (split ratio 31:1); detection 2.0 kV negative ESI–ion-trap MS; solutes *1* glucose, *2* maltose, *3* maltotriose, *4* maltotetraose, *5* maltopentaose, *6* maltohexaose, *7* maltoheptaose; sample concentrations **a** 20 µg/mL, **b** 4 µg/mL

Gradient elution of carbohydrates under HILIC conditions is a useful method for efficient separation. The homologs of glucose up to maltoheptaose were separated on the 200T-PAAm within 16 min using a linear gradient of 90–60% CH_3CN within 15 min, and were detected by a negative ESI (2 kV) using single MS full scan mode over the range of 150 to 2,000 *m/z*. As shown in Fig. 5a, carbohydrate oligomers up to heptamers were separated with acceptable efficiency. All peaks were observed in split form due to anomerization, but no significant peak broadening or tailing was observed. To obtain the mass chromatogram, 50 nL of carbohydrate sample solution at 20 µg/mL concentration was injected directly into the column, and thus 1 ng of sample on column could be detected. The same volume of sample solution at 4 µg/mL concentration was similarly separated by the same system to show that 200 pg of each sample on-column could be detected as shown in Fig. 5b, in spite of the single MS full scan mode on wide range. The peak widths were about 0.5 min for the combined two peaks of anomers for each saccharide and seemed to be narrower than those in the report obtained using a capillary column packed with TSK-GEL Amide-80 particles (100 mm×75 µm ID, 5 µm) LC-ESI-MS for larger *N*-glycans with molecular weights over 1,000 [16]. However, it is difficult to compare the separation efficiency of these systems in detail, since a gradient elution was used in the separation. Similarly, all peak widths in the chromatogram were broadened over 1 min in a test run carried out for the separation of the same sample (20 µg/mL) using a particle-packed column, PolyHydroxyethyl A (PolyLC, Columbia, MD, USA, 100 mm×320 µm ID) with a linear gradient over 15 min of 80–60% CH_3CN/13 mM NH_4OCOCH_3 (pH=5.5) by a negative ESI (3.3 kV). The analysis system of oligosaccharides using particle-packed columns resulted in broad peaks with around 1 min widths [52]. These results indicate that the monolithic capillary column 200T-PAAm in

HILIC LC-ESI-MS mode can provide better separation efficiency than the reported particle-packed columns. The injection of 50 nL of sample into a monolithic silica column of 100 μm ID using an injector valve resulted in a severe loss of separation efficiency due to the extra-column effect at the injection site. The use of a monolithic silica column of 200 μm ID can reduce the loss of column efficiency caused by large-volume samples [47]. In general, capillary chromatography provides better detection sensitivity than HPLC using conventional-sized, 4.6 mm ID columns probably due to the favorable spraying properties without flow splitting [53, 54]. According to these findings, the monolithic silica column of 200 μm ID with a higher phase ratio seems to be a promising platform for high-efficiency separation and the sensitive detection of the underivatized carbohydrate oligomers by HILIC for LC separation.

To determine the limits of detection of the present system, four different sample solutions of sucrose, maltose, and trehalose were examined by the 200T-PAAm-ESI-MS/MS system. Following the first step of the IT-MS, the precursor ion of m/z=401, $[M+AcO]^-$ was selected and these ions were then submitted to selected reaction monitoring (SRM) of the m/z=341, $[M-H]^-$ ion, which was formed by the second fragmentation. This method is widely employed to determine the LOD of MS detection [55, 56]. Figure 6a shows the separation using a 50 nL sample of 400 ng/mL (1.17 mM) carbohydrates. The sample solution was diluted fivefold for each chromatogram, and was supplied to the LC-MS/MS system. Maltose was not detected at 16 ng/mL (46 nM), but sucrose and trehalose were detected even at 3.2 ng/mL (9.4 nM), as shown in Fig. 6d. Since the injection volume in each experiment was 50 nL, the LOD of the system at S/N=3 can be estimated at 470 attomol or lower for nonreducing oligosaccharides, sucrose and trehalose.

From Figs. 1–6, the separation efficiency of the 200T-PAAm is better than that of column packed with 5 μm particles employed in previous studies [16, 52, 56]. As described in several reports, the advantages of the monolithic silica support over the particle-packed columns is the high permeability to reduce column backpressure, and high separation efficiency caused by the small silica skeletons [43–47]. In the case of HILIC mode, the modification method by an on-column polymerization is useful without

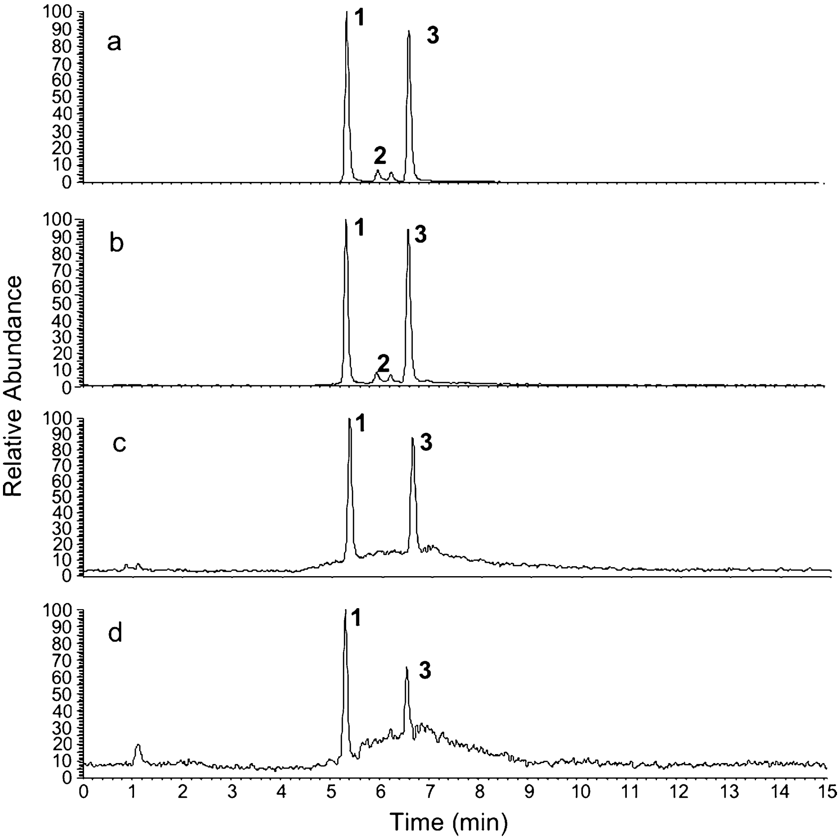


Fig. 6 LC-ESI-MS/MS SRM chromatogram of a mixture of disaccharides. Mobile phase 90–70% CH_3CN (13 mM NH_4OCOCH_3) in 10 min, and 70–40% CH_3CN (13 mM NH_4OCOCH_3) in 10.1 min linear gradients; flow ratio 0.2 mL/min; column 200T-PAAm, 267 mm×200 μm ID; linear velocity 4.2 mm/s (split ratio 31:1); detection 2.0 kV negative ESI–ion-trap MS/MS, m/z=401, SRM at 341; solutes *1* sucrose, *2* maltose, *3* trehalose; sample concentrations **a** 400 ng/mL, **b** 80 ng/mL, **c** 16 ng/mL, and **d** 3.2 ng/mL

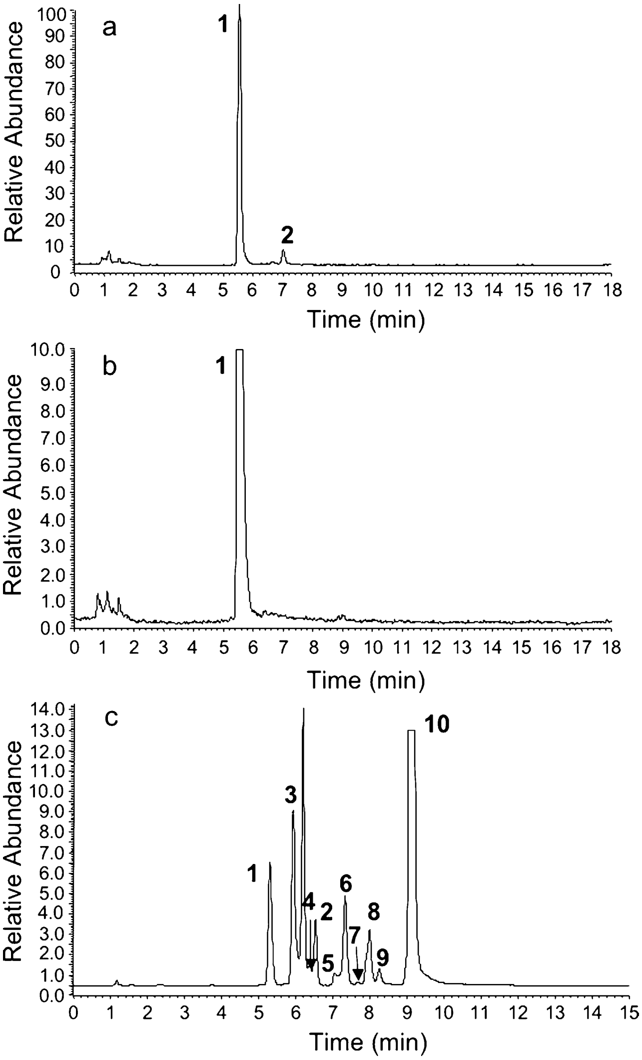


Fig. 7 LC-ESI-MS/MS SRM chromatogram of plant extracts using the same separation-detection conditions as Fig. 6. Samples **a** a corn extract, **b** a soybean extract, and **c** an *Arabidopsis thaliana* leaf extract; peaks *1* sucrose, *2* trehalose, *3* maltose (α and β isomers), *4–10* unknown disaccharides

loss of the original separation efficiency of the silica support [44, 57–60]. It is more difficult to obtain good particulate columns for the HILIC mode versus the RP mode. Additionally, the polymer-coating method can increase the phase ratio of the monolithic columns effectively, and supplies enough retentivity of the analytes for better separation efficiency [59]. The availability and performance of particulate columns for HILIC mode is still limited [7]. The present system has several advantages for the analysis of native carbohydrates such as: (1) the use of a highly efficient 200 µm ID column reduces the extra-column effect caused by the injection of a sample, and represents a good compromise for LC-ESI-MS analysis; and (2) the sensitivity (subfemtomol for underivatized carbohydrates) of LC-MS/MS detection is kept high owing to the reduced sample dilution based on the higher efficiency. Carbohydrates derivatized with a fluorescence tag could be detected at the 100 attomol level [61], but it is difficult to apply this method to nonreducing saccharides, since it is based on Schiff base formation of the aldehyde functionality of the saccharides. In the present system, nonreducing saccharides showed a lower LOD due to the absence of peak broadening caused by anomerization.

The analysis for nonreducing oligosaccharides by the HILIC-ESI-MS/MS system was applied to the separation and detection of several carbohydrates from plant extracts. Figure 7a shows a mass chromatogram of a corn extract. Using an SRM (m/z=341) method, trehalose was detected in the presence of a much higher concentration of sucrose. On the other hand, no disaccharide except for sucrose was detected in the case of a soybean extract, as shown in Fig. 7b. When the same HILIC-ESI-MS/MS analysis was applied to a leaf extract of *Arabidopsis thaliana*, the presence of sucrose, maltose, and trehalose was confirmed. In addition, at least six disaccharides (m/z=341, $[\mathrm{M-H}]^-$) were detected as shown in Fig. 7c. Adequate reproducibility was obtained for several runs under the same conditions. The combination of the high reproducibility of the HPLC system and the highly sensitive detection of the ESI-MS/MS system will be helpful for plant metabolomics. For a highly sensitive detection in LC-ESI-MS system, it is also important to reduce ion-suppression, and an improvement in separation efficiency is known to be effective [62].

Conclusions

A novel HILIC-ESI-MS(/MS) system using a monolithic silica capillary column modified with polyacrylamide was developed and tested for the highly efficient separation and the highly sensitive detection of underivatized carbohydrates. The theoretical plate height H in the LC-ESI system is 19–22 µm, providing N=12,000–14,000 even at a high linear velocity, u=7.6 mm/s, although this column produces H=7 µm under the optimum conditions. For underivatized carbohydrates, the limits of detection were shown to be subfemtomolar owing to the advantageous point of using a capillary column, in which the effect of sample dilution is significantly lower than a microbore column system. Only 50 nL of sample is introduced into the LC-MS(/MS) system, so this methodology will be helpful to identify highly polar components included in the scarce biological samples.

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References

1. Takahashi N (1996) J Chromatogr A 720:217–225
2. El Rassi Z, Nashabeh W (1995) Carbohydrate analysis: high performance liquid chromatography and capillary electrophoresis. Elsevier, Amsterdam, pp 267–360
3. Linden JC, Lawhead CL (1975) J Chromatogr 105:125–133
4. Kahle V, Tesarík K (1980) J Chromatogr 191:121–128
5. Hase S, Koyama S, Daiyasu H, Takemoto H, Hara S, Kobayashi Y, Kyogoku Y, Ikenaka T (1986) J Biochem 100:1–10
6. Alpert AJ (1990) J Chromatogr 499:177–196
7. Hemström P, Irgum K (2006) J Sep Sci 29:1784–1821
8. Björklund M, Hearn MTW (1996) J Chromatogr A 728:149–169
9. Alpert AJ, Shukla M, Shukla AK, Zieske LR, Yuen SW, Ferguson MAJ, Mehlert A, Pauly M, Orlando R (1994) J Chromatogr A 676:191–202
10. Churms SC (1991) Carbohydrates, vol II (CRC handbook of chromatography). CRC, Boston, p 307
11. Churms SC (1996) J Chromatogr A 720:75–91
12. Takegawa Y, Deguchi K, Ito H, Keira T, Nakagawa H, Nishimura S (2006) J Sep Sci 29:2533–2540
13. Yoshida T (1997) Anal Chem 69:3038–3043
14. Karlsson G, Winge S, Sandberg H (2005) J Chromatogr A 1092:246–249
15. Charlwood J, Birrell H, Bouvier ESP, Langridge J, Camilleri P (2000) Anal Chem 72:1469–1474
16. Wuhrer M, Koeleman CAM, Deelder AM, Hokke CH (2004) Anal Chem 76:833–838
17. Davies MJ, Smith KD, Carruthers RA, Chai W, Lawson AM, Hounsell EF (1993) J Chromatogr 646:317–326
18. Ito S, Kawasaki N, Ohta M, Hyuga M, Hyuga S, Hayakawa T (2002) J Chromatogr A 968:89–100
19. Schulz BL, Packer NH, Karlsson NG (2002) Anal Chem 74:6088–6097
20. Barroso B, Dijkstra R, Geerts M, Lagerwerf F, van Veelen P, de Ru A (2002) Rapid Commun Mass Spectrom 16:1320–1329
21. Pesek JJ, Matyska MT (2005) J Sep Sci 28:1845–1854
22. Pesek JJ, Matyska MT, Larrabee S (2007) J Sep Sci 30:637–647
23. El Rassi Z (1999) Electrophoresis 20:3134–3144
24. Liu Y, Shu C, Lamb D (1997) J Capil Electrophor 4:97–103
25. Zemann A, Nguyen DT, Bonn G (1997) Electrophoresis 17:1142–1147
26. Soga T, Heiger DN (1998) Anal Biochem 261:73–78
27. Sato S, Soga T, Nishioka T, Tomita M (2004) Plant J 40:151–163
28. Voegel PD, Zhou W, Baldwin RP (1997) Anal Chem 69:951–957
29. Johnson DC (1986) Nature 321:451–452
30. Swinney K, Pennington J, Bornhop DJ (1999) Analyst 124:221–225
31. Harvey D (2001) J Proteomics 1:311–328
32. Bahr U, Pfenninger A, Karas M, Stahl B (1997) Anal Chem 69:4530–4535
33. Suzuki S, Honda S (1998) Electrophoresis 19:2539–2560
34. Hofstetter-Kuhn S, Paulus A, Gassmann E, Widmer HM (1991) Anal Chem 63:1541–1547
35. O'Shea TJ, Lunte SM, LaCourse WR (1993) Anal Chem 65:948–951
36. LaCourse WR, Owens GS (1996) Electrophoresis 17:310–318
37. Ye J, Baldwin RP (1993) Anal Chem 65:3525–3527
38. Fermier AM, Colón LA (1996) J High Resol Chromatogr 19:613–616
39. Klockow A, Paulus A, Figueiredo V, Amado R, Widmer HM (1994) J Chromatogr A 644:412–415
40. Xu X, Kok WT, Poppe H (1995) J Chromatogr A 716:231–240
41. Kakehi K, Kinoshita M, Hayase S, Oda Y (1999) Anal Chem 71:1592–1596
42. Soga T, Ohashi Y, Ueno Y, Naraoka H, Tomita M, Nishioka T (2003) J Proteome Chem 2:488–494
43. Hara T, Kobayashi H, Ikegami T, Nakanishi K, Tanaka N (2006) Anal Chem 78:7632–7642
44. Ikegami T, Fujita H, Horie K, Hosoya K, Tanaka N (2006) Anal Bioanal Chem 386:578–585
45. Motokawa M, Kobayashi H, Ishizuka N, Minakuchi H, Nakanishi K, Jinnai H, Hosoya K, Ikegami T, Tanaka N (2002) J Chromatogr A 961:53–63
46. Tanaka N, Kobayashi H, Nakanishi K, Minakuchi H, Ishizuka N (2001) Anal Chem 73:420A–429A
47. Ikegami T, Dicks E, Kobayashi H, Morisaka H, Tokuda D, Cabrera K, Hosoya K, Tanaka N (2004) J Sep Sci 27:1292–1302
48. SeQuant (2007) A practical guide to HILIC 11. SeQuant, Umeå; see also http://www.sequant.com/sn/p_details.php?id=7. Accessed 5 Feb 2008
49. Desmet G, Clicq D, Gzil P (2005) Anal Chem 77:4058–4070
50. Tosoh Bioscience (2007) TSK-GEL application note: TSK-GEL Amide-80 normal phase chromatography columns for the separation of saccharides. http://www.separations.us.tosohbioscience.com/NR/rdonlyres/41B82826-9C84-4EBE-80A9-9BD80C9E21D7/0/L_NPC_AN_Amide80_02.pdf. Accessed 5 Feb 2008
51. Nishikawa T, Suzuki S, Kubo H, Ohtani H (1996) J Chromatogr A 720:167–172
52. Cappiello A, Trufelli H, Famiglini G, Pierini E, Capellacci S, Penna A, Ricci F, Ingarao C, Penna N (2007) Water Res 41:2911–2920
53. Yue GH, Luo QZ, Zhang J, Wu SL, Karger BL (2007) Anal Chem 79:938–946
54. Luo QZ, Shen YF, Hixson KK, Zhao R, Yang F, Moore RJ, Mottaz HM, Smith RD (2005) Anal Chem 77:5028–5035
55. Matuszewski BK, Constanzer ML, Chavez-Eng CM (2003) Anal Chem 75:3019–3030
56. Ding L, Xie F, Zhao M, Wang S, Xie J, Xu G (2007) J Sep Sci 30:35–41
57. Ikegami T, Horie K, Jaafar J, Hosoya K, Tanaka N (2007) J Biochem Biophys Methods 70:31–37
58. Horie K, Ikegami T, Hosoya K, Saad N, Fiehn O, Tanaka N (2007) J Chromatogr A 1164:198–205
59. Núñez O, Ikegami T, Kajiwara W, Miyamoto K, Horie K, Tanaka N (2007) J Chromatogr A 1156:35–44
60. Preinerstorfera B, Lubda D, Lindner W, Lämmerhofer M (2006) J Chromatogr A 1106:94–105
61. Liu JP, Shirota O, Novotny M (1991) Anal Chem 63:413–417
62. Tolstikov VV, Lommen A, Nakanishi K, Tanaka N, Fiehn O (2003) Anal Chem 75:6737–6740